Evaluation of Purinergic Mechanism for the Treatment of Voiding Dysfunction: A Study in Conscious Spinal Cord-injured Rats

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Background: To investigate the effect of a selective $P2X_3$–$P2X_{2/3}$ purinergic receptor antagonist (A-317491) on detrusor hyperreflexia in conscious chronic spinal cord-injured female rats.

Methods: Six chronic spinal cord-transected female Sprague-Dawley rats (290–336 g) were used in this study. Spinal transection at the T8–T9 segmental level was performed using aseptic techniques under halothane anesthesia. Fourteen to 16 weeks after spinal transection, A-317491, a selective $P2X_3$ purinergic receptor antagonist, was administered intravenously in cystometry studies at increasing doses of 0.03, 0.1, 0.3, 1, 3, 10 and 30 μmol/kg at 40–50 minute intervals. Cystometrograms (CMGs) were performed before and after the administration of each dose of the drug.

Results: The continuous filling of CMGs revealed a large number of small-amplitude (> 8 cmH_2O), non-voiding contractions (NVCs) (average, 9.7 per voiding cycle) preceding voiding contractions (mean amplitude, 31 cmH_2O; duration, 2.5 minutes), which occurred at an interval of 539 seconds and at a pressure threshold of 5.7 cmH_2O. When tested in a range of doses (0.03–30 μmol/kg, intravenous), A-317491 in doses between 1 and 30 μmol/kg significantly ($p < 0.05$) increased the interval between voids by 25%, reduced the number of NVCs by 42–62%, and increased the pressure threshold for voiding by 53–73%, but did not change the amplitude of the duration of the voiding contractions. The effects of the drug were apparent within 10 minutes following administration.

Conclusion: These results indicate that purinergic mechanisms, presumably involving $P2X_3$ or $P2X_{2/3}$ receptors on bladder C-fiber afferent nerves, play an important role in the detrusor hyperreflexia that occurs after spinal cord injury in rats. [*J Chin Med Assoc* 2007;70(10):439–444]

Key Words: afferent nerve, detrusor overactivity, spinal cord injury, urinary bladder

There is no conflict of interest between the authors and the pharmaceutical company that provided the A-317491.

Introduction

Spinal cord injury (SCI) rostral to the lumbosacral level causes lower urinary tract dysfunctions, including detrusor hyperreflexia, incontinence, autonomic dysreflexia and detrusor-sphincter dyssynergia.[1–6] Intravesical or systemic administration of capsaicin or resiniferatoxin in SCI cats and rats reduced detrusor hyperreflexia, uninhibited bladder contractions and vesicovascular reflexes, indicating that these disorders are triggered, at least in part, by capsaicin-sensitive C-fiber bladder afferents. These afferents also appear to contribute to neurogenic bladder dysfunction in humans, since the intravesical administration of capsaicin or resiniferatoxin in patients with SCI or multiple sclerosis decreases detrusor hyperactivity and the number of incontinence episodes.

In normal animals, a considerable proportion of bladder C-fiber afferents are chemosensitive but insensitive to mechanical stimuli (i.e. silent C-fiber afferents) and are, therefore, unresponsive to bladder distension

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and make no contribution to micturition reflexes. However, following SCI, the electrical and morphologic properties of bladder afferent neurons change. The afferents become mechanosensitive and can initiate bladder reflexes. It has been speculated that neurotrophic factors, such as the nerve growth factor or other chemicals released in the bladder, may be responsible for the afferent nerve plasticity after SCI.

Among the various chemicals known to modulate the activity of bladder afferent nerves, adenosine triphosphate (ATP) has recently been the subject of much attention. A subpopulation of C-fiber bladder afferent nerves exhibit $P2X_2$ and $P2X_3$ purinergic receptors[7–9] and are excited by exogenous ATP administered intravesically by *in vivo* preparations or when applied to the bladder *in vitro*. ATP, which is released from the urothelium by mechanical or chemical stimuli, may act on the C-fiber afferents that are located adjacent to or within the urothelium and, thereby, induce reflex bladder activity. Mice in which the $P2X_3$ receptor has been knocked out exhibited hypoactive bladders[10–12] and decreased bladder excitatory responses to the intravesical administration of ATP. Because the $P2X_3$ receptors are expressed exclusively in afferent nerves, these data suggest that purinergic afferent mechanisms play an important role in reflex voiding in mice.

In the present study, we examined the effect of a recently developed potent and selective non-nucleotide $P2X_3$ and $P2X_{2/3}$ purinergic receptor antagonist (A-317491) on voiding function in chronic spinal cord-injured rats to determine if ATP contributes to neurogenic detrusor overactivity.

Methods

Spinal cord transection and care of SCI rats

Six chronic spinal cord transected female Sprague-Dawley rats (290–336 g) were used in this study. Spinal transections at the T8–T9 segmental level were performed using aseptic techniques under halothane anesthesia. After a T9–T10 laminectomy, the dura matter was cut and the spinal cord transected with scissors, after which the point of a 16-gauge needle was moved 6 times around the inner surface of the exposed vertebra to ensure complete transection. Then, a piece of sterile sponge (Gelfoam; The Upjohn Company, Kalamazoo, MI, USA) was placed between the 2 cut ends of the spinal cord and the overlying muscle and skin were sutured. The rats were kept in a room in which the temperature was maintained at 23–23.5°C, and they were treated with antibiotics (ampicillin 150 mg/kg, intramuscular) every 2 days for 10 days. The bladders were manually compressed 2 or 3 times daily after spinal transection to prevent over-distension of the bladder and infection. Perigenital stimulation with a cotton swab was used to activate a somatovesical reflex pathway and promote bladder emptying.[13]

Cystometric studies

Fourteen to 16 weeks after spinal transaction, cystometry was performed on the same animals. The rats were anesthetized with 2% halothane, and then the jugular vein was cannulated for drug and fluid administration. The bladder was exposed via a midline abdominal incision. The bladder end of a polyethylene catheter (inner diameter 0.76 mm, outer diameter 1.22 mm; Clay-Adams, Parsippany, NJ, USA) was heated to create a collar and passed through a small incision at the bladder dome. A 3-0 surgical silk suture (Ethicon, Somerville, NJ, USA) was tightened around the collar of the catheter. The cystostomy catheter was placed inside a larger polyethylene tube that acted as a tunnel to prevent the bending of the cystostomy catheter during the experiment. After the closure of the abdominal wound, the rats were placed in a restraining cage (Ballman Cage) in a normal crouched posture and allowed to recover from the halothane anesthesia for 1–2 hours before the experiment. The cystostomy catheter was connected to an infusion pump (model 55-4150; Harvard Apparatus, Holliston, MA, USA) to allow the continuous infusion of physiologic saline at room temperature into the bladder. Bladder pressure was monitored during cystometry by connecting the bladder catheter to a Statham pressure transducer. Continuous cystometrograms (CMGs) were performed using a constant infusion (0.2 mL/min) of saline into the bladder to induce repetitive voiding.[14] The infusion of saline continued for at least 2 hours before the control voiding parameters were measured. Several control CMGs were performed on the same animal. Body temperature was monitored and maintained within the range of 36–38°C through an external heating device.

Drug administration

A-317491, a selective $P2X_3$ purinergic receptor antagonist, was administered intravenously in the cystometry studies, at increasing doses of 0.03, 0.1, 0.3, 1, 3, 10 and 30 μmol/kg at 40–50 minute intervals. CMGs were measured before and after the administration of each dose of the drug.

Statistical analysis

The quantitative data are presented as mean ± standard error. Wilcoxon signed rank test was used to compare each parameter before and after drug administration.

A value of $p < 0.05$ was taken to indicate a significant difference.

Results

Control urodynamic measurements

During the surgical procedure to insert the cystostomy catheter to conduct the CMGs, it was clear that all of the SCI rats exhibited severely distended and hypertrophied bladders. CMGs revealed a large number of small-amplitude ($> 8\ cmH_2O$) non-voiding contractions (NVCs) (average, 9.7 per voiding cycle) during the saline infusion. Voiding contractions (mean amplitude, 31 cmH_2O; duration, 2.5 minutes) occurred at an average interval of 539 seconds, equivalent to approximately 1.8 mL of infused saline between voids. The average pressure threshold for inducing voiding contractions was 5.7 cmH_2O. The mean bladder capacity was calculated from the sum of the residual volume and infusion volume, which was 2.5 mL during CMGs. Repeated CMGs performed over 1–2 hour intervals in the same animal yielded reproducible filling and voiding parameters.

Effect of A-317491 on voiding and bladder activity

After obtaining control CMGs, A-317491 was administered intravenously in increasing doses, from 0.03 to 30 μmol/kg. The interval between voiding contractions (intercontractile interval) did not significantly change after smaller doses of the drug (0.03–0.3 μmol/kg), but increased significantly ($p < 0.05$) after 1–30 μmol/kg of A-317491 (from 539 to 655–676 seconds) (Figures 1 and 2). The mean number of small-amplitude ($\geq 8\ cmH_2O$) NVCs which occurred between the voiding contractions significantly decreased after 1–30 μmol/kg of the drug had been administered (from 9.72 to 5.6, 4.6, 4.75 and 3.63, respectively, $p < 0.05$), but did not change significantly following smaller doses (0.03–0.3 μmol/kg) (Figures 1 and 3). The pressure threshold for inducing voiding increased after 1–30 μmol/kg of A-317491 (from 5.73 to 8.76, 9.91, 9.74 and 9.72, respectively, $p < 0.05$), but was not significantly changed following smaller doses (Figures 1 and 4). The amplitude, duration of voiding contractions and baseline bladder pressure were not affected by any doses of the drug (Figures 5 and 6). The effect of A-317491 was apparent within 10–15 minutes after administration, and, after the largest dose of the drug, persisted for the duration of the experiment (2–3 hours).

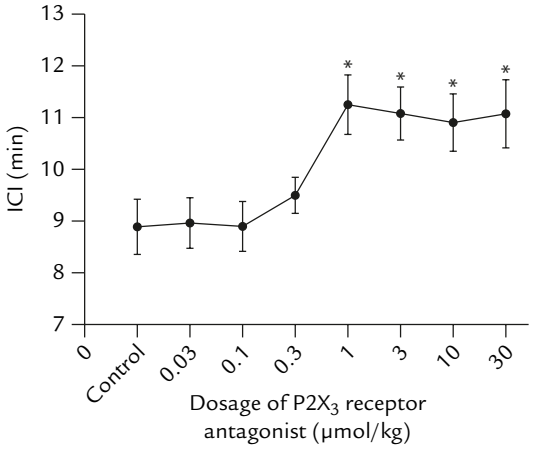

Figure 2. Intervals of effective contraction (intercontractile interval, ICI) before and after intravenous $P2X_3$ receptor antagonist administration in cystometry study ($n = 6$). *$p < 0.05$.

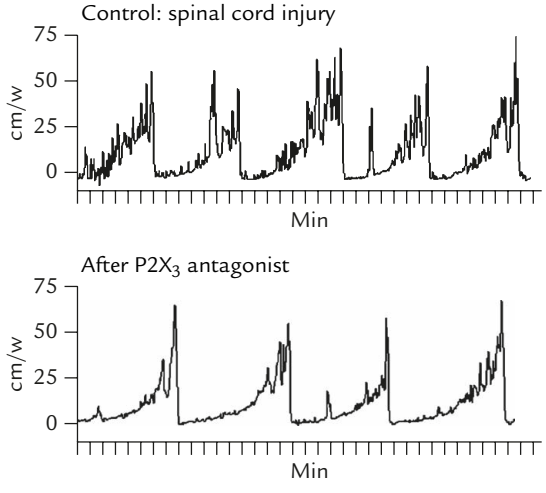

Figure 1. Cystometrograms showing the effects of A-317491 on voiding and bladder activity.

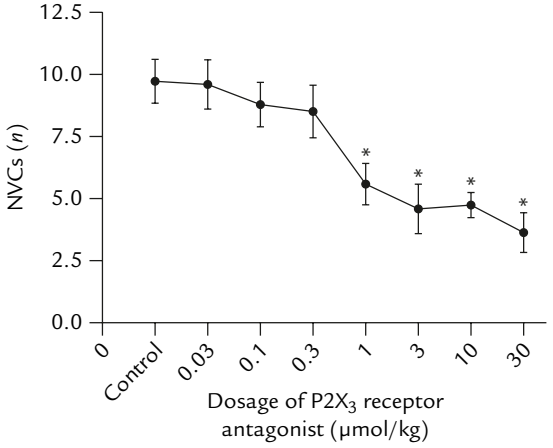

Figure 3. Number of non-voiding contractions (NVCs) before and after intravenous $P2X_3$ receptor antagonist administration in cystometry study ($n = 6$). *$p < 0.05$.

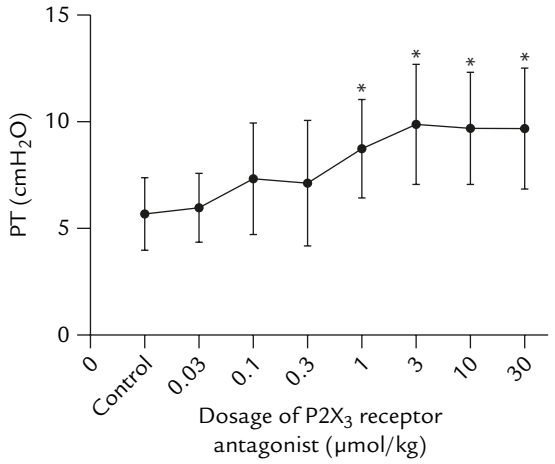

Figure 4. Pressure threshold (PT) before and after intravenous $P2X_3$ receptor antagonist administration in cystometry study ($n = 6$). $*p < 0.05$.

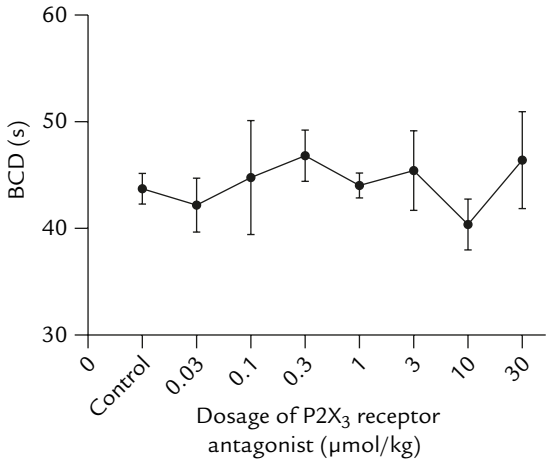

Figure 6. Duration of effective bladder contraction (BCD) before and after intravenous $P2X_3$ receptor antagonist administration in cystometry study ($n = 6$).

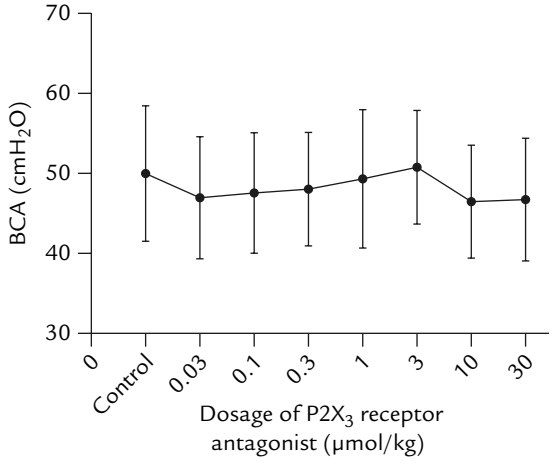

Figure 5. Amplitude of effective bladder contraction (BCA) before and after intravenous $P2X_3$ receptor antagonist administration in cystometry study ($n = 6$).

Discussion

The present experiments revealed that the systemic administration of A-317491, a $P2X_3$–$P2X_{2/3}$ receptor antagonist, during continuous fast-infusion cystometry in conscious, chronic SCI rats reduced the number of non-voiding bladder contractions, increased the intravesical pressure threshold for inducing voiding and also increased the interval between voids without altering the amplitude or duration of the voiding contractions. The drug also decreased the frequency of voiding and increased the volume of urine per void without affecting total urine excretion under conditions of natural bladder filling in SCI animals held in a metabolism cage. These results indicate that purinergic mechanisms or $P2X_3$ or $P2X_{2/3}$ receptors on bladder afferent nerves play an important role in detrusor hyperreflexia due to SCI.

A-317491 is a non-nucleotide purinergic receptor antagonist that competitively blocks recombinant human and rat $P2X_3$ and $P2X_{2/3}$ receptors with a 100-fold selectivity over other types of P2X receptors. The drug exhibits very weak or no affinity for a large selection of other cell surface receptors and ion channels. Recent studies reveal that the drug also blocked native $P2X_3$ and $P2X_{2/3}$ receptors in rat dorsal root ganglion cells and dose-dependently reduced complete Freund's adjuvant-induced thermal hyperalgesia ($ED_{50} = 30$ μmol/kg, subcutaneous), attenuating both the thermal hyperalgesia and mechanical allodynia ($ED_{50} = 10$–15 μmol/kg, subcutaneous) following chronic nerve constriction injury. These data, coupled with other reports indicating the presence of $P2X_{2/3}$ receptors in IB4-positive C-fiber afferent neurons, indicate that purinergic mechanisms are involved in the peripheral and central sensitization of C-fiber nociceptors in chronic inflammatory and neuropathic pain. In the present experiments, similar doses of A-317491 reduced the frequency of voiding as well as the pressure threshold for inducing voiding without affecting maximal micturition pressure. This suggests that the drug suppressed the afferent limb of the spinal micturition reflex without altering the efferent limb of the reflex or the properties of the bladder detrusor muscle.

The CMG measurements in SCI female rats in the present study were similar to those obtained previously in awake SCI female rats. The most prominent changes following SCI were an increase in bladder capacity

(2.5 mL *vs.* 1 mL in awake spinal cord-intact rats) and the presence of large numbers of NVCs prior to micturition. Although these changes were probably due to the spinal injury, it is also possible that some changes were related to the abdominal surgery and insertion of the bladder catheter on the day of the experiment or the fast infusion rate in cystometry.

The chronic transection of the thoracic spinal cord induces significant changes in the cystometric parameters of conscious female rats, including the appearance of uninhibited bladder contractions before the onset of micturition and obstructed voiding and decreased voiding efficiency.[15] The present study reveals that A-317491 was effective in increasing the intercontractile interval and volume threshold, and in decreasing the number of NVCs in long-term SCI rats. However, the drug did not alter the amplitude and duration of voiding bladder contractions. This indicates that the drug did not alter the contractility of the detrusor muscle or the efferent pathways to the bladder.

Cockayne et al[12] reported that conscious or anesthetized $P2X_3$-null mice also had significantly decreased micturition frequencies and increased bladder capacities, although their voiding bladder pressures were not reduced. These data suggest that the $P2X_3$-null mice had a sensory rather than a motor defect in the bladder.

The rat bladders in this study were hypertrophied and enlarged following long-term SCI. It has been proposed that animals with a larger volume threshold tend to have a higher pressure threshold, indicating that the more distended bladders require a higher intramural tension to stimulate the afferent fibers, or that higher levels of afferent firing are needed to trigger a micturition reflex. However, the pressure threshold before treatment in this study was lower than that of the normal rats (5.73 *vs.* 13 cmH_2O).[16] This indicates that the micturition reflex is triggered more easily due to the increased excitability of the afferent nerves, despite the hypertrophied and enlarged bladders following SCI.

In the present study, CMGs were performed in conscious rats. It was reported that urethane affected the central transmitter mechanisms involved in the micturition reflex and decreased the voiding efficiency and volume threshold for inducing micturition in normal rats.[17,18] Urethane was also reported to completely depress reflex micturition in chronic SCI rats.[19] Thus, SCI rats appear to increase the sensitivity of their micturition reflex to urethane.[20] Therefore, it would be better to test the CMGs in conscious awake animals in this study rather than urethane-anesthetized ones.

The bladder catheter was inserted through a dome in this study, which may have eliminated the impact of bladder outlet obstruction produced by a transurethral catheter.[21] Nevertheless, the disadvantages of this transvesical catheterization method may be the possible irritation of the bladder, and the limitation of bladder movement during filling and voiding. However, this irritation change was not evident during the first day following transvesical catheterization. Therefore, this procedure is best for acute experiments immediately after catheter implantation[22] or experiments pursued at least 6–7 days after implantation.[23] Thus, the results of the CMGs that we performed on the same day as the transvesical catheterization in this study should be reliable.

Some previous studies measured the voided volume and residual volume, and subsequently calculated the bladder capacity and voiding efficiency[24,25] of SCI rats. However, the measurements of voided volume and residual volume are relatively subjective, and it is difficult to measure these parameters accurately for conscious animals in the Ballman cage. Actually, Yoshiyama et al also found that bladder capacity varied considerably from 0.33 to 3.3 mL within the SCI group.[24] Nevertheless, in the present study, the rat bladders appeared consistently hypertrophied and enlarged in all animals. Thus, we did not measure the voided volume or the residual volume in this experiment.

Previous clinical studies reported that the intravesical administration of capsaicin or resiniferatoxin produced clinical improvements in neurogenic bladder conditions, such as incontinence and detrusor hyperreflexia, in SCI and multiple sclerosis patients,[26–29] as well as hypersensitive bladder dysfunction in interstitial cystitis.[30] This study revealed that A-317491 inhibited reflex bladder hyperactivity including non-voiding detrusor contractions without affecting voiding contractility, which is similar to the delayed suppressed effects elicited by intravesical capsaicin or resiniferatoxin administration. Thus, A-317491 may provide a new alternative treatment for the above pathologic conditions. The possibility of oral administration is the obvious advantage of A-317491 over intravesical instillation of capsaicin or resiniferatoxin. In clinical terms, the intravesical administration of capsaicin can produce suprapubic pain, burning, macroscopic hematuria, or increased incontinence during the first week after treatment.[30] Therefore, after conducting further clinical trials to validate the efficacy and side effects, A-317491 may be a better treatment of choice for neurogenic bladder disorders related to bladder afferent excitability, such as SCI, multiple sclerosis and interstitial cystitis.

References

1. Yoshiyama M, Nezu FM, Yokoyama O, Chancellor MB, de Groat WC. Influence of glutamate receptor antagonists on micturition in rats with spinal cord injury. *Exp Neurol* 1999; 159:250–7.
2. de Seze M, Wiart L, de Seze MP, Soyeur L, Dosque JP, Blajezewski S, Moore N, et al. Intravesical capsaicin versus resiniferatoxin for the treatment of detrusor hyperreflexia in spinal cord injured patients: a double-blind, randomized, controlled study. *J Urol* 2004;171:251–5.
3. Christensen P, Bazzocchi G, Coggrave M, Abel R, Hultling C, Krogh K, Media S, et al. A randomized, controlled trial of transanal irrigation versus conservative bowel management in spinal cord-injured patients. *Gastroenterology* 2006;131:738–47.
4. Osgood SL, Kuczkowski KM. Autonomic dysreflexia in a parturient with spinal cord injury. *Acta Anaesth Belgica* 2006; 57:161–2.
5. Elliott S, Krassioukov A. Malignant autonomic dysreflexia in spinal cord injured men. *Spinal Cord* 2006;44:386–92.
6. Karsenty G, Reitz A, Wefer B, Boy S, Schurch B. Understanding detrusor sphincter dyssynergia: significance of chronology. *Urology* 2005;66:763–8.
7. Studeny S, Torabi A, Vizzard MA. P2X2 and P2X3 receptor expression in postnatal and adult rat urinary bladder and lumbosacral spinal cord. *Am J Physiol* 2005;289:R1155–68.
8. North RA. Molecular physiology of P2X receptors. *Physiol Rev* 2002;82:1013–67.
9. Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* 2000;163:2002–7.
10. Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, et al. P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J Physiol* 2005;567:621–39.
11. de Groat WC. The urothelium in overactive bladder: passive bystander or active participant? *Urology* 2004;64(Suppl):7–11.
12. Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, et al. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 2000;407:1011–5.
13. Kruse MN, de Groat WC. Consequences of spinal cord injury during the neonatal period on micturition reflexes in the rat. *Exp Neurol* 1994;125:87–92.
14. Yoshiyama M, de Groat WC, Fraser MO. Influences of external urethral sphincter relaxation induced by alpha-bungarotoxin, a neuromuscular junction blocking agent, on voiding dysfunction in the rat with spinal cord injury. *Urology* 2000;55: 956–60.
15. Seki S, Erickson KA, Seki M, Nishizawa O, Igawa Y, Ogawa T, de Groat WC, et al. Elimination of rat spinal neurons expressing neurokinin 1 receptors reduces bladder overactivity and spinal c-fos expression induced by bladder irritation. *Am J Physiol* 2005;288:F466–73.
16. Yoshiyama M, Nezu FM, Yokoyama O, de Groat WC, Chancellor MB. Changes in micturition after spinal cord injury in conscious rats. *Urology* 1999;54:929–33.
17. Yoshiyama M, Roppolo JR, de Groat WC. Effects of LY215490, a competitive alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist, on the micturition reflex in the rat. *J Pharmacol Exp Ther* 1997;280:894–904.
18. Yoshiyama M, de Groat WC. Supraspinal and spinal alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and N-methyl-D-aspartate glutamatergic control of the micturition reflex in the urethane-anesthetized rat. *Neuroscience* 2005;132: 1017–26.
19. Cheng CL, de Groat WC. The role of capsaicin-sensitive afferent fibers in the lower urinary tract dysfunction induced by chronic spinal cord injury in rats. *Exp Neurol* 2004;187:445–54.
20. Kruse MN, Bennett B, De Groat WC. Effect of urinary diversion on the recovery of micturition reflexes after spinal cord injury in the rat. *J Urol* 1994;151:1088–91.
21. Yokoyama O, Yoshiyama M, Namiki M, de Groat WC. Influence of anesthesia on bladder hyperactivity induced by middle cerebral artery occlusion in the rat. *Am J Physiol* 1997; 273:R1900–7.
22. Yokoyama O, Ishiura Y, Komatsu K, Mita E, Nakamura Y, Kunimi K, Morikawa K, et al. Effects of MK-801 on bladder overactivity in rats with cerebral infarction. *J Urol* 1998; 159:571–6.
23. Yoshiyama M, de Groat WC. Effect of bilateral hypogastric nerve transection on voiding dysfunction in rats with spinal cord injury. *Exp Neurol* 2002;175:191–7.
24. Yoshiyama M, deGroat WC, Fraser MO. Influences of external urethral sphincter relaxation induced by alpha-bungarotoxin, a neuromuscular junction blocking agent, on voiding dysfunction in the rat with spinal cord injury. *Urology* 2000;55:956–60.
25. Lazzeri M, Spinelli M, Zanollo A, Turini D. Intravesical vanilloids and neurogenic incontinence: ten years experience. *Urol Int* 2004;72:145–9.
26. Kim JH, Rivas DA, Shenot PJ, Green B, Kennelly M, Erickson JR, O'Leary M, et al. Intravesical resiniferatoxin for refractory detrusor hyperreflexia: a multicenter, blinded, randomized, placebo-controlled trial. *J Spinal Cord Med* 2003;26: 358–63.
27. Fowler CJ. Intravesical treatment of overactive bladder. *Urology* 2000;55(Suppl):60–4.
28. Lazzeri M, Spinelli M, Beneforti P, Zanollo A, Turini D. Intravesical resiniferatoxin for the treatment of detrusor hyperreflexia refractory to capsaicin in patients with chronic spinal cord diseases. *Scand J Urol Nephrol* 1998;32:331–4.
29. Chen TY, Corcos J, Camel M, Ponsot Y, Tu le M. Prospective, randomized, double-blind study of safety and tolerability of intravesical resiniferatoxin (RTX) in interstitial cystitis (IC). *Int Urogynecol J* 2005;16:293–7.
30. Chancellor MB, de Groat WC. Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder. *J Urol* 1999;162:3–11.